Federal Democratic Republic of Ethiopia

EDICT OF GOVERNMENT

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ET ISO 12872 (2012) (English): Olive oils and olive-pomace oils -- Determination of the 2-glyceryl monopalmitate content

ISO INSIDE

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ETHIOPIAN
STANDARD

ES ISO 12872:2012

First edition

Olive oils and olive-pomace oils- Determination of the 2-glyceryl monopalmitate content

(Identical with ISO 12872:2010)

ICS: 67.200.10

Published by Ethiopian Standards Agency

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Foreword

This Ethiopian Standard has been prepared under the direction of the Technical Committee for Animal and vegetables fats and oils (TC 23) and published by the Ethiopian Standards Agency (ESA).

The standard is identical with ISO 12872:2010, Olive oils and olive-pomace oils - Determination of the 2-glyceryl monopalmitate content, published by International Organization for Standardization (ISO).

For the purpose of this Ethiopian Standard, the adopted text shall be modified as follows.

- The phrase “International Standard” shall be read as “Ethiopian Standard”; and
- A full stop (.) shall substitute comma (,) as decimal marker.

Olive oils and olive-pomace oils — Determination of the 2-glyceryl monopalmitate content

1 Scope

This International Standard specifies a procedure for the determination of the content, as a percentage mass fraction, of 2-glyceryl monopalmitate in olive oils and olive-pomace oils that are liquid at ambient temperature (20 °C).

NOTE This International Standard is based on COI/T.20/Doc. 23:2006[6].

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
2-glyceryl monopalmitate content
mass fraction of 2-glyceryl monopalmitate in the monoacylglycerol fraction, determined according to the method specified in this International Standard

NOTE The 2-glyceryl monopalmitate content is expressed as a percentage.

4 Principle

The oil, after suitable preparation, is subjected to the action of pancreatic lipase. A partial hydrolysis takes place that is specific for positions 1 and 3 of the triacylglycerol molecule so that 2-monoacylglycerols are obtained as reaction products. The percentage of 2-glyceryl monopalmitate in the monoacylglycerol fraction is determined, after silylation, by capillary gas chromatography.

5 Reagents

WARNING — Comply with any local regulations which specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade, and distilled or demineralized water or water of equivalent purity.

5.1 Silica gel, with a particle size of 0,063 mm to 0,200 mm (70/280 mesh), prepared as follows: put the silica gel into a porcelain cup, dry in an oven at 160 °C for 4 h, then cool at ambient temperature in a desiccator. Add a volume of water equivalent to 5 % of the mass of the silica gel as follows: weigh 152 g of silica gel into a 500 ml Erlenmeyer flask, add 8 g of water, stopper and homogenize carefully. Leave to settle for at least 12 h before using.

5.2 *n*-Hexane, chromatography grade.

5.3 Isopropanol.

5.4 Isopropanol-water mixture, volume fractions 50 ml/100 ml.

5.5 Pancreatic lipase, activity between 2,0 and 10 lipase units per milligram (see Annex C).

5.6 Buffer solution of tris(hydroxymethyl)aminomethane: prepare an aqueous solution (1 mol/l) with pH 8 and mix with concentrated HCl, volume fractions 50 ml/100 ml.

5.7 Sodium cholate, special enzyme grade, aqueous solution, mass fraction 0,1 g/100 g.

Use this solution within 15 days of preparation.

5.8 Calcium chloride, aqueous solution, mass fraction 22 g/100 g.

5.9 Diethyl ether, chromatography grade.

5.10 Elution solvent: mixture of *n*-hexane-diethyl ether, volume fraction of *n*-hexane 87 ml/100 ml and of diethyl ether 13 ml/100 ml.

5.11 Sodium hydroxide, aqueous solution, mass fraction 12 g/100 g.

5.12 Phenolphthalein, ethanolic solution, mass concentration 1 g/100 ml.

5.13 Carrier gas: hydrogen or helium, gas chromatography grade.

5.14 Auxiliary gases: hydrogen, free from moisture and organic substances, and synthetic air, gas chromatography grade.

5.15 Silylation reagent: mixture of pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS); volume fractions: 9 ml/13 ml, 3 ml/13 ml, and 1 ml/13 ml, respectively.

5.16 Reference samples: pure monoacylglycerols and mixtures of monoacylglycerols with a known composition similar to that of the sample.

6 Apparatus

Usual laboratory equipment and in particular the following.

6.1 Erlenmeyer flasks, of capacity 25 ml.

6.2 Beakers, of capacities 100 ml, 250 ml, and 300 ml.

6.3 Glass chromatography column, 21 mm to 23 mm internal diameter, 400 mm in length, with septum and stopcock.

6.4 Measuring cylinders, of capacities 10 ml, 50 ml, 100 ml, and 200 ml, ISO 4788[2] class A.

6.5 Round-bottomed flasks, of capacities 100 ml and 250 ml.

6.6 Rotary evaporator.

6.7 Centrifuge tubes, conical bottom, of capacity 10 ml, with ground-glass stopper.

6.8 Centrifuge, suitable for 10 ml and 100 ml tubes.

6.9 Water bath, capable of maintaining a temperature of (40 ± 0,5) °C.

6.10 Graduated pipettes, of capacities 1 ml and 2 ml, ISO 835[1] class A.

6.11 Hypodermic syringe, 1 ml.

6.12 Microsyringe, 100 µl.

6.13 Separating funnel, 1 000 ml.

6.14 Gas chromatograph, suitable for use with capillary columns, equipped with the components specified in 6.14.1 to 6.14.5.

6.14.1 Cold on-column injector.

6.14.2 Flame ionization detector.

6.14.3 Column oven, capable of maintaining the temperature to within ± 1 °C.

6.14.4 Computer-based integration system.

6.14.5 Fused silica capillary column, of length 8 m to 12 m and internal diameter 0,25 mm to 0,32 mm, coated with methylpolysiloxane or 5 % phenyl methylpolysiloxane, with a film thickness of 0,10 µm to 0,30 µm, suitable for use at 370 °C.

6.15 Microsyringe, 10 µl, with hardened needle, of minimum length 7,5 cm in length, suitable for on-column injection.

7 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555[3].

It is important that the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 661. Oils with a free acidity greater than 3 % require a neutralization step as specified here.

Introduce 50 g of the oil into a 1 000 ml separating funnel (6.13) and dissolve it in 200 ml of *n*-hexane (5.2). Add 100 ml of isopropanol (5.3) and a volume of sodium hydroxide solution (5.11) corresponding to the free acidity of the oil plus an excess of 5 %. Shake vigorously for 1 min, add 100 ml of water, shake again and leave to settle.

After separation, remove the lower soapy layer. Also remove any intermediate layer of mucilage and insoluble matter which often forms. Wash the *n*-hexane solution of the oil with successive 50 ml to 60 ml portions of the isopropanol-water mixture (5.4) until the washed phase is neutral to phenolphthalein (5.12).

Remove most of the hexane by distillation under vacuum, e.g. by using a rotary evaporator (6.6), and transfer the oil to a 100 ml round-bottomed flask (6.5). Dry it under vacuum to complete solvent removal.

By the end of this procedure, ensure that the acidity of the oil is below 0,5 %.

9 Procedure

9.1 Preparatory steps

9.1.1 Introduce 1,0 g of the oil, pretreated (Clause 8) where necessary, into a 25 ml Erlenmeyer flask (6.1) and dissolve it in 10 ml of elution solvent (5.10). Leave the solution to settle for at least 15 min before starting the silica gel column chromatography procedure.

If the solution is cloudy, centrifuge it to ensure optimal conditions for chromatography.

Ready-to-use 500 mg silica gel solid phase extraction (SPE) cartridges may be used.

9.1.2 Fill the chromatography column (6.3) with about 30 ml of elution solvent (5.10), insert a wad of cotton wool at the bottom of the column using a glass rod; press to remove the air.

In a beaker, prepare a suspension of 25 g of silica gel (5.1) in about 80 ml of elution solvent and transfer it to the column by means of a funnel.

Make sure that all the silica gel is transferred to the column; wash with elution solvent, open the stopcock and allow the solvent to drain until the solvent level is about 2 mm above the silica gel.

9.2 Column chromatography

9.2.1 Conventional procedure

Weigh 1,0 g of prepared test sample (9.1.1), to the nearest 0,1 mg, into a 25 ml Erlenmeyer flask (6.1).

Dissolve the sample in 10 ml of elution solvent (5.10). Transfer the solution on to the chromatography column (9.1.2). Avoid moving the surface of the column.

Open the stopcock and let the sample solution drain until it reaches the level of the silica gel. Elute with 150 ml of elution solvent. Regulate the flow to a rate of 2 ml/min (150 ml shall pass through the column in about 60 min to 70 min).

Collect the eluate in a previously weighed 250 ml round-bottomed flask (6.5). Evaporate the solvent under vacuum and remove the last traces of solvent under a stream of nitrogen.

Weigh the flask and calculate the mass recovered by difference.

9.2.2 Procedure when ready-to-use silica SPE cartridges are employed

Introduce 1 ml of the solution (9.1.1) into the cartridges, previously conditioned with 3 ml of *n*-hexane (5.2). After percolation of the solution, elute with 4 ml of the *n*-hexane-diethyl ether elution solvent (5.10). Recover the eluate in a 10 ml tube and evaporate to dryness under a stream of nitrogen. Subject the dry residue to the action of pancreatic lipase (5.5). Check the fatty acid composition before and after passage through the SPE cartridge.

9.3 Hydrolysis with pancreatic lipase

9.3.1 Weigh 0,1 g of the oil into a centrifuge tube (6.7). Add 2 ml of buffer solution (5.6), 0,5 ml of sodium cholate solution (5.7) and 0,2 ml of calcium chloride solution (5.8), shaking well after each addition. Cover the tube with the glass stopper and place it in the water bath (6.9) at (40 ± 0,5) °C.

9.3.2 Add 20 mg of lipase (5.5), mix carefully (to avoid wetting the stopper) and place in the water bath for exactly 2 min, then remove, shake vigorously for exactly 1 min and cool.

9.3.3 Add 1 ml of diethyl ether (5.9), stopper and shake vigorously, then centrifuge and transfer the ether solution to another clean, dry tube by means of a microsyringe.

9.4 Preparation of the silylated derivatives and gas chromatography

Take 100 µl of solution (9.3.3) using a microsyringe and transfer to a 10 ml tube with a conical bottom (6.7). Eliminate the solvent under a gentle stream of nitrogen, add 200 µl of silylation reagent (5.15), stopper the tube and leave to settle for 20 min.

After 20 min, add 5 ml of *n*-hexane (5.2). The solution is then ready for gas chromatography.

9.5 Gas chromatography

9.5.1 Operating conditions

The following operating conditions are recommended:

a) injector temperature: below solvent boiling point (68 °C);

b) detector temperature: 350 °C;

c) oven temperature programming: isothermal at 60 °C for 1 min; then increase at a rate of 15 °C/min to 180 °C, then at a rate of 5 °C/min to 340 °C; finally maintain at 340 C° for 20 min;

d) carrier gas: hydrogen or helium, regulated to the suitable linear speed in order to obtain the resolution shown in Annex A;

e) retention time of triacylglycerol C_{54}: (40 ± 5) min (see Figure A.2);

f) injection volume: 0,5 µl to 1 µl of the solution obtained in 9.4.

Optimize the separation conditions to get the required resolution. Ensure that the height of the peak for 2-glyceryl monopalmitate is at least 10 % of the full-scale value.

9.5.2 Identification of peaks

The individual monoacylglycerols are identified by comparing the retention times obtained with those obtained for standard monoacylglycerol mixtures analysed under the same test conditions.

9.5.3 Quantitative evaluation

The area of each peak is calculated by electronic integration.

10 Expression of results

The content of glyceryl monopalmitate, w_{MP}, expressed as a percentage mass fraction, is calculated from the ratio between the area of the relevant peak and the sum of the peak areas of all the monoacylglycerols (see Figure A.2) as follows:

$$w_{\mathrm{MP}} = \frac{A_{\mathrm{MP}}}{\sum A} \times 100$$

where

A_{MP} is the peak area of the glyceryl monopalmitate peak;

ΣA is the sum of the peak areas of all monoacylglycerols.

Report the result to one decimal place.

11 Precision

11.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

11.2 Repeatability

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, should, in not more than 5 % of cases, exceed the values of r given in Table B.1.

11.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, should, in not more than 5 % of cases, exceed the values of R given in Table B.1.

12 Test report

The test report shall include at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard (ISO 12872:2010);

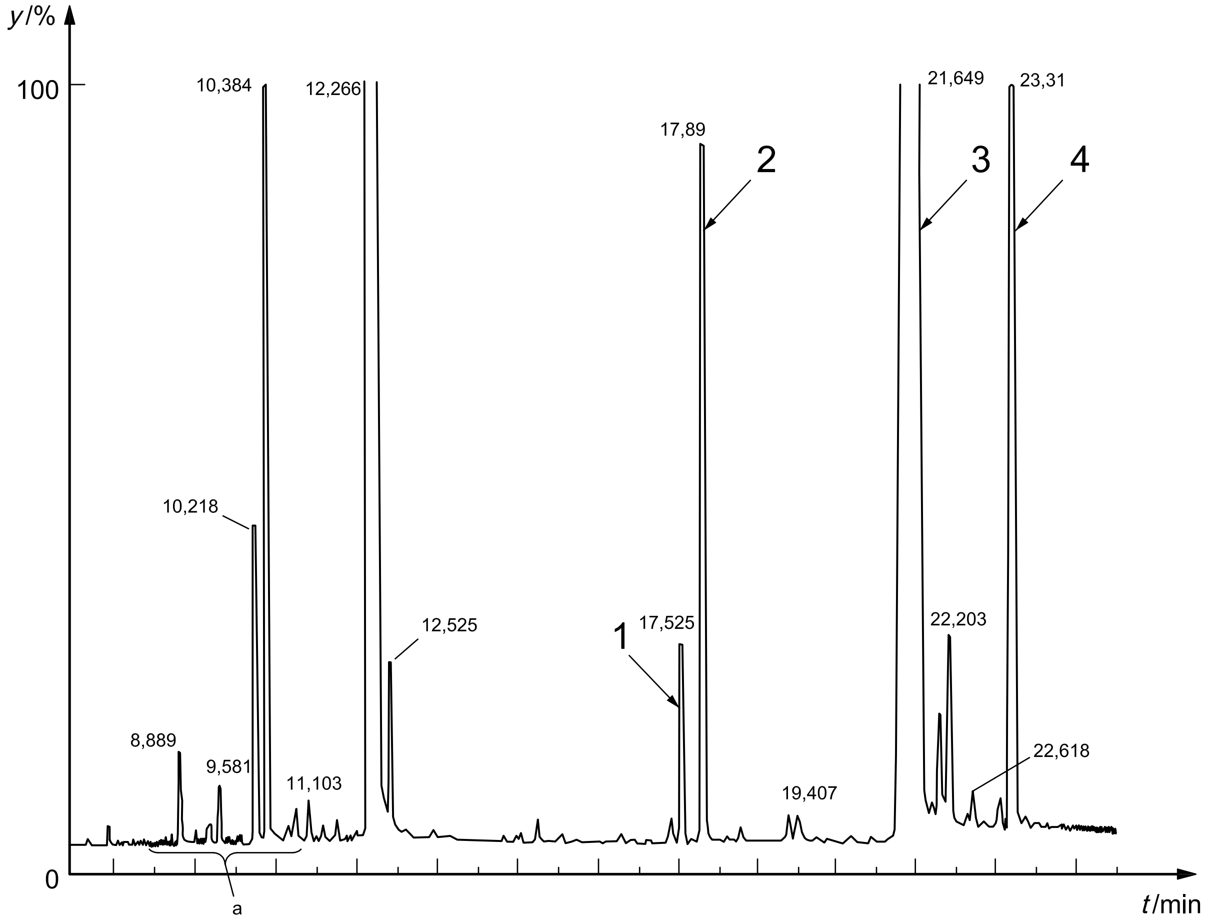
d) the result(s) obtained;

e) if the repeatability has been checked, the final quoted result obtained;

f) any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s).

Annex A
(informative)

Chromatograms



Key

1 2-monopalmitoleine

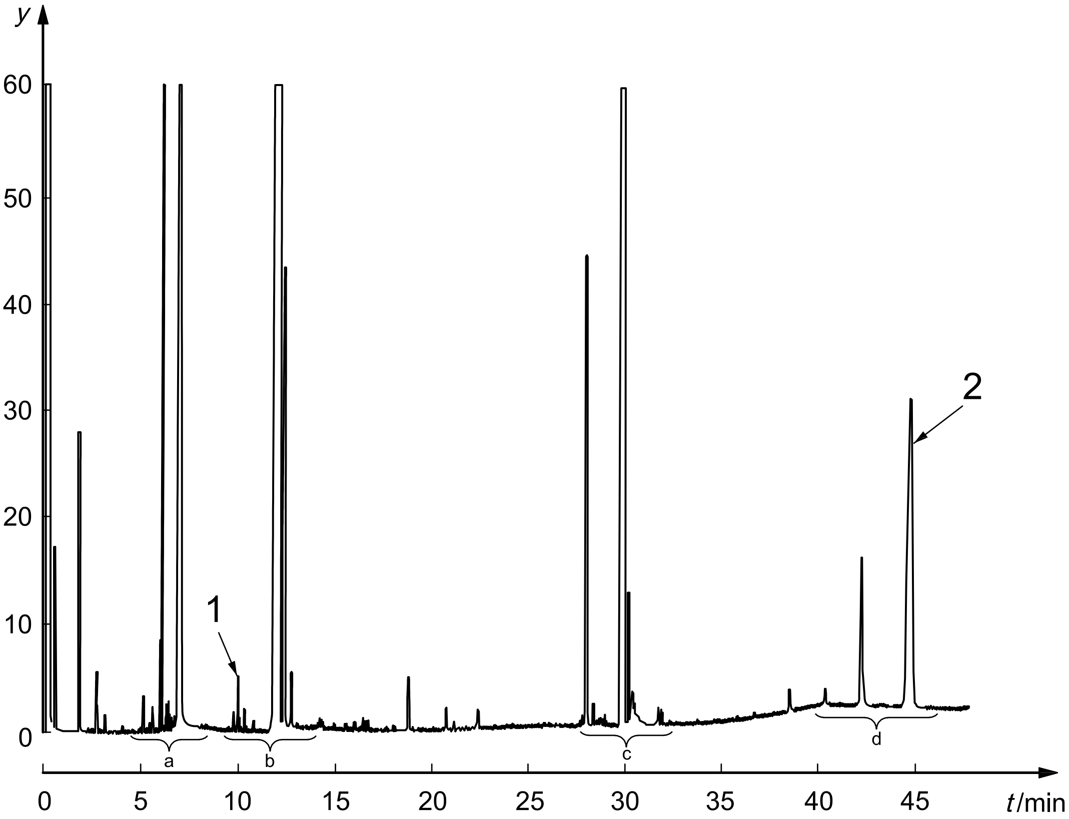
2 2-monopalmitine

3 2-mono C_{18} unsaturated

4 squalene

t	time
y	measure of relative peak size
a	Free fatty acids.

Figure A.1 — Chromatogram of the silylation reaction products obtained by lipase action on a refined olive oil spiked with 20 % volume fraction esterified oil



Key

1	2-monopalmitate	t	time
2	triacylglycerol C_{54}	y	measure of relative peak size, arbitrary units

NOTE In these conditions (capillary column 8 m to 12 m), the wax fraction elutes with the diacylglycerol fraction or soon after it. After lipase action, triacylglycerol content should not exceed 15 % mass fraction.

[a] Free fatty acids.

[b] Monoacylglycerols.

[c] Diacylglycerols.

[d] Triacylglycerols.

Figure A.2 — Genuine olive oil after the action of the lipase and after silylation

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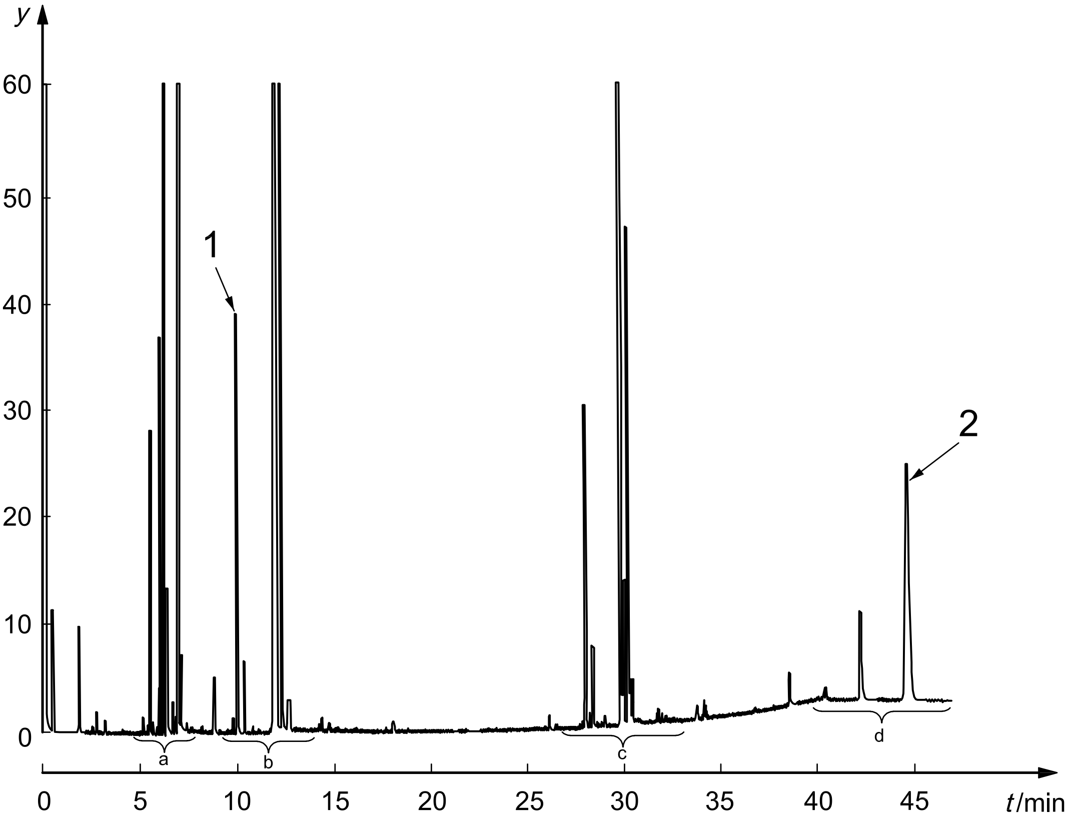

Key

1 2-monopalmitate

2 triacylglycerol C_{54}

t time

y measure of relative peak size, arbitrary units

NOTE In these conditions (capillary column 8 m to 12 m), the wax fraction elutes with the diacylglycerol fraction or soon after it. After lipase action, triacylglycerol content should not exceed 15 % mass fraction.

[a] Free fatty acids.

[b] Monoacylglycerols.

[c] Diacylglycerols.

[d] Triacylglycerols.

Figure A.3 — An esterified oil after the action of the lipase and silylation

Annex B
(informative)

Results of an interlaboratory test

The precision data in Table B.1 are derived from the results of an international collaborative trial.

The interlaboratory test was organized by the International Olive Council and was coordinated from the University of Genoa. Participants included 12 laboratories from five countries.

The test results were subjected to statistical analysis in accordance with ISO 5725-1[4] and ISO 5725-2[5] to give the precision data shown in Table B.1. Outliers were examined by applying the tests of Cochran and Grubbs to the laboratory results for each determination (replicates a and b) and each sample.

Table B.1 — Statistical results

Parameter	**Sample**				
	A Extra virgin olive oil	**B** Lampante virgin olive oil	**C** Refined olive oil	**D** Refined olive oil and re-esterified oil (90+10) % volume fractions	**E** Refined olive oil and re-esterified oil (80+20) % volume fractions
No. participating laboratories, n_P	12	12	12	12	12
No. laboratories retained after eliminating outliers, n_p	12	12	12	12	12
No. test results in all laboratories, n_t	24	24	24	24	24
Mean glyceryl monopalmitate content, $\overline{w}_\mathrm{MP}$, % mass fraction	0,46	0,75	0,90	1,81	2,82
Repeatability standard deviation, s_r, %	0,04	0,04	0,06	0,04	0,09
Coefficient of variation of repeatability, $C_{V,\,r}$, %	8,91	5,44	6,77	1,95	3,32
Repeatability limit, r, %	0,11	0,11	0,17	0,10	0,26
Reproducibility standard deviation, s_R, %	0,05	0,10	0,09	0,20	0,31
Coefficient of variation of reproducibility, $C_{V,\,R}$, %	11,07	12,66	10,16	11,07	10,88
Reproducibility limit, R, %	0,14	0,27	0,26	0,56	0,86

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Annex C
(informative)

Preparation and activity of the lipase

C.1 Supplementary reagents

C.1.1.1 **Acetone**, anhydrous.

C.1.1.2 **Gum Arabic solution**, 100g/l.

C.1.1.3 **Sodium cholate**, special enzyme grade solution, 0,2 g/ml.

C.1.1.4 **Sodium hydroxide solution**, 0,1 mol/l.

C.2 Preparation of the lipase

Lipase is available commercially, but may also be prepared in the laboratory as follows.

Take 5 kg of fresh pig pancreas that has been chilled to 0 °C. Remove the surrounding solid fat and connective tissue and triturate in a blender to obtain a fluid paste. Stir this paste for 4 h to 6 h with 2,5 l of acetone (C.1.1.1) and centrifuge. Extract the residue three times more with the same volume of acetone, then twice with a mixture of acetone and diethyl ether (5.9) (volume fraction 50 ml/100 ml), and twice with diethyl ether.

Dry the residue under vacuum for 48 h to obtain a stable powder. When stored in the refrigerator and kept away from damp, it keeps for a long time.

C.3 Checking of the lipase activity

Prepare an olive oil emulsion as follows.

Shake a mixture of 165 ml of gum Arabic solution (C.1.1.2), 15 g of crushed ice, and 20 ml of neutralized olive oil in a suitable agitator for 10 min.

In a 50 ml beaker, place 10 ml of this emulsion, followed successively by 0,3 ml of sodium cholate solution (C.1.1.3) and 20 ml of water.

Put the beaker into an oven thermostatically maintained at 37 °C; insert the electrodes of a pH-meter and a spiral stirrer.

By means of a burette, add sodium hydroxide solution (C.1.1.4), drop by drop, until the pH reaches 8,3.

Add an aqueous suspension of the lipase being checked (mass concentration 0,1 g/ml of lipase). Check the pH. As soon as the pH reaches 8,3, start the stopwatch and drip in the sodium hydroxide solution at such a rate as to maintain the pH at 8,3. Note down the volume of solution consumed every minute.

Record the observations in graph form, plotting the time readings on the abscissa and the volume, in millilitres, of sodium hydroxide solution required to maintain the pH constant on the ordinate. A linear graph should be obtained.

The activity, A, of the lipase, measured in lipase units per milligram, is given by:

$$A = \frac{q_V \ c \times 1\,000}{m}$$

where

q_V is the rate of consumption, in millilitres per minute, of the sodium hydroxide solution (calculated from the graph);

c is the amount of substance concentration, in moles per litre, of the sodium hydroxide solution (0,1 mol/l);

m is the mass, in milligrams, of the lipase used in the test.

The lipase unit is defined as the amount of enzyme that liberates 10 microequivalents of acid per minute.

Bibliography

[1] ISO 835, *Laboratory glassware — Graduated pipettes*

[2] ISO 4788, *Laboratory glassware — Graduated measuring cylinders*

[3] ISO 5555, *Animal and vegetable fats and oils — Sampling*

[4] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[5] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[6] COI/T.20/Doc. 23:2006, *Method for the determination of the percentage of 2-glyceryl monopalmitate*

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Ethiopian Standards Agency

Organization and Objectives

The Ethiopian Standards Agency (ESA) is the national standards body of Ethiopia established in 2010 based on regulation No. 193/2010.ESA is established due to the restructuring of Quality and Standards Authority of Ethiopia (QSAE) which was established in 1970.

ESA's objectives are:-

- ❖ Develop Ethiopian standards and establish a system that enable to check weather goods and services are in compliance with the required standards,
- ❖ Facilitate the country's technology transfer through the use of standards,
- ❖ Develop national standards for local products and services so as to make them competitive in the international market.

Ethiopian Standards

The Ethiopian Standards are developed by national technical committees which are composed of different stakeholders consisting of educational Institutions, research institutes, government organizations, certification, inspection, and testing organizations, regulatory bodies, consumer association etc. The requirements and/ or recommendations contained in Ethiopian Standards are consensus based that reflects the interest of the TC representatives and also of comments received from the public and other sources. Ethiopian Standards are approved by the National Standardization Council and are kept under continuous review after publication and updated regularly to take account of latest scientific and technological changes.
Orders for all Ethiopian Standards, International Standard and ASTM standards, including electronic versions, should be addressed to the Documentation and Publication Team at the Head office and Branch (Liaisons) offices. A catalogue of Ethiopian Standards is also available freely and can be accessed in from our website.

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International Involvement
ESA, representing Ethiopia, is a member of the International Organization for Standardization (ISO), and Codex Alimentarius Commission (CODEX). It also maintains close working relations with the international Electro-technical Commission (IEC) and American Society for Testing and Materials (ASTM).It is a founding member of the African Regional Organization for standardization (ARSO).

More Information?
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